The Effects Of Avian Influenza NS1 Protein On Interferon Promoter Activation

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Tiivistelmä – Referat – Abstract

Influenza A viruses are pathogens infecting birds and selected mammals. They are responsible for around 500 000 human deaths each year and pose a substantial economic burden to the healthcare system. The most important pathway in influenza virus detection is a retinoic acid-inducible gene I pathway, which recognizes the 5'-triphosphate in viral RNA. Its activation leads to the production of interferons: a group of cytokines important in overcoming viral infection. In order to replicate successfully, viruses had to develop mechanisms to overcome host defences. They include, among others, regulation of interferons and interferon stimulated genes expression. During influenza A virus infection, this function is performed by viral non-structural protein 1 (NS1). The aim of this study was evaluating the effect of NS1 of five different avian influenza strains and one seasonal influenza strain on activation of type I and III interferon gene promoters. The NS1 of seasonal virus H3N2 shown the highest suppression of both interferon I and III promoters, while NS1 originating from avian H9N2 and H7N7 strains had limited effect on interferon promoter activation. NS1 of H5N1/04, H5N1/97 and H7N9 was very effective at suppressing interferon type I promoter, which correlates with the severity of the infection in humans. When it comes to interferon type III promoter, H7N9 was very efficient at the suppression, while NS1 of H5N1/04 had little impact on promoter activation. The study has provided more information on the efficiency of potentially pandemic avian influenza strains at inhibition of interferon response and may be a base for further research. The project was conducted at the Finnish Institute of Health and Welfare.

Avainsanat – Nyckelord – Keywords

avian influenza, H5N1, H7N9, H9N2, H7N7, H3N2, interferons, RIG-I, NS1

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1. Abbreviations

AIVs	Avian influenza viruses
BSA	Bovine serum albumin
CMV	Cytomegalovirus
CPS	Counts per second
CPSF30	Cleavage and polyadenylation specificity factor 30
CTT	C-terminal tail
dsRNA	Double-stranded RNA
EAGLE-MEM	Eagle's minimum essential medium
ED	Effector domain
FFluc	Firefly luciferase
HA	Hemagglutinin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HP	Highly pathogenic
HPAIV	Highly pathogenic avian influenza virus
HRP	Horseradish peroxidase
IAVs	Influenza A viruses
IBVs	Influenza B viruses
IFNAR	Interferon-α/β receptor
IFNLR	IFN-λ receptor
IFNs	Interferons
IKKα	Inhibitor of nuclear factor kappa B kinase alpha
IRF3	Interferon regulatory factor 3
ISGs	Interferon stimulated genes
ISRE	Interferon-sensitive response elements
IκB	Inhibitor of nuclear factor kappa B
JAK	Janus kinase
LD	Linker domain
LP	Low pathogenic
LPAIV	Low pathogenic avian influenza virus
M1	Matrix protein 1
M2	Matrix protein 2
MxA	Myxovirus resistance gene A
NA	Neuraminidase
NEP	Nuclear export protein
NES	Nuclear export signal
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	Nuclear localization signal
NP	Nucleoprotein
NS1	Non-structural protein -1

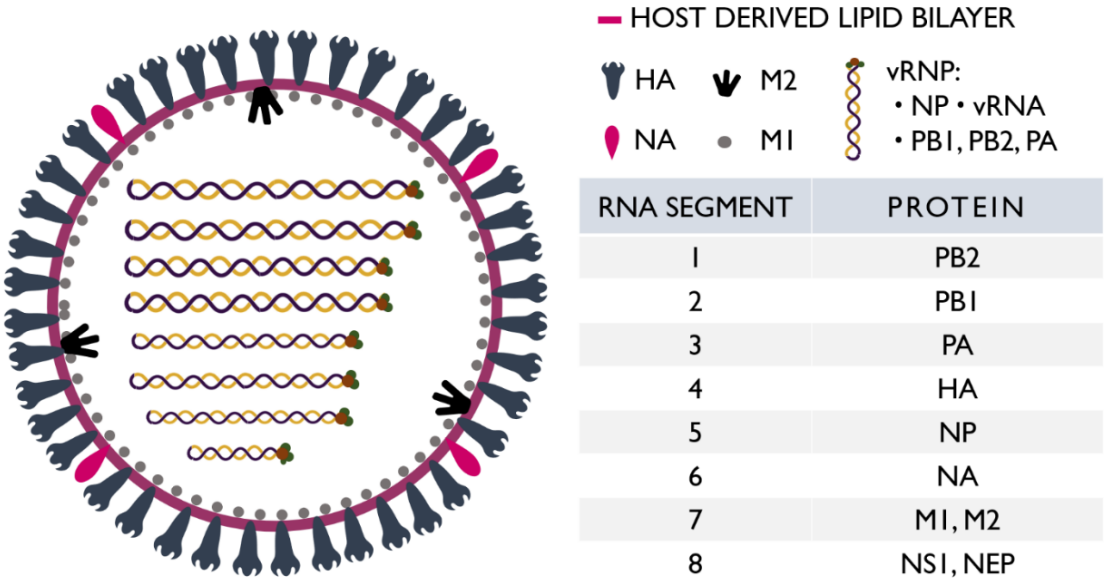
OAS	2'-5' oligo (A) synthetase
PA	Polymerase acidic
PAB II	Poly(A)-binding protein II
PACT	Protein activator of the interferon-induced PKR
PAMPs	Pathogen associated molecular patterns
PB1	Polymerase basic 1
PB2	Polymerase basic 2
Pi3K	Phosphoinositide 3-kinase
PKR	RNA-dependent protein kinase
PRRs	Pattern recognition receptors
PVDF	Polyvinylidene difluoride
RBD	RNA-binding domain
RIG-I	Retinoic acid-inducible gene I
Rluc	Renilla luciferase
RNPs	Ribonucleoproteins
SA	Sialic acid
STAT	Signal transducer and activator of transcription
SV	Sendai virus
TBK1	TANK-binding kinase 1
TRIM25	Tripartite motif-containing protein 25
vRNA	Viral RNA
vRNP	Viral ribonucleoprotein
WHO	World Health Organization

2. Introduction

2.1. Influenza virus classification and structure

Influenza viruses belong to the family of *Orthomyxoviridae* and are classified based on differences in nucleoprotein (NP) into several genera, including influenza A, B, C, and D viruses. Human infections are usually caused either by influenza A (IAVs) or B viruses (IBVs), but infections with influenza C viruses has also been reported (Mostafa et al., 2018). While IBVs are thought to be restricted to humans and seals, IAVs infect a wide range of species (Osterhaus et al., 2000), their most important natural reservoir being aquatic birds (Webster, Monto & Braciale 2013; Mostafa et al., 2018). Currently, two seasonal IAVs are circulating in humans: H3N2 and H1N1. However, some avian influenza viruses can occasionally infect humans with a very high mortality rate (World Health Organisation [WHO], 2018b).

The genome of influenza A viruses consists of hair-pin shaped, negative-sense single-stranded RNA divided into eight segments (Fig. 1). Each segment encodes one or more proteins and is coated by multiple viral NPs, as well as a polymerase complex comprised of polymerase basic 2 (PB2), polymerase basic 1 (PB1) and polymerase acidic (PA) encoded consecutively by first three segments. These ribonucleoproteins (RNPs) are enclosed by a matrix protein layer consisting of matrix protein 1 (M1) and matrix protein 2 (M2) which functions as an ion channel. The entire particle is also surrounded by host-derived lipid bilayer containing two types of viral surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). HA is responsible for binding the host cell receptors and membrane fusion, while NA is necessary for the release of the virus. These glycoproteins differ between strains and affect their ability to bind cell receptors. In fact, IAVs are classified based on HA and NA subtypes. IAVs also encode for several non-structural proteins which have different functions in infected host cells, two most important ones being non-structural protein 1 (NS1) and nuclear export protein (NEP) (Hsu, 2018).



RNA SEGMENT	PROTEIN
1	PB2
2	PB1
3	PA
4	HA
5	NP
6	NA
7	M1, M2
8	NS1, NEP

Figure 1. Structure of IAV. The virion is enveloped by host cell derived lipid bilayer and two types of glycoproteins: HA and NA. The nucleocapsid is formed by structural M1 protein and ion channels (M2 protein). Inside there are eight viral RNAs (vRNAs) encoding PB2, PB1, PA, HA, NP, NA, M1 and M2, NS1 and NEP accordingly. Each segment is coated with NP, PB1, PB2 and PA, creating viral ribonucleoprotein (vRNP) (Krammer et al., 2018; modified).

2.2. Viral entry and replication

In order to understand why influenza outbreaks occur, it is important to know how the virus spreads and replicates (Fig. 2). IAV infection is characterized by symptoms such as fever, sore throat, cough, runny nose, headaches and fatigue. Although the main target in humans is epithelial cells of the respiratory tract, the virus is also able to infect dendritic cells and macrophages (Westenius et al., 2014). The modes of transmission for IAVs include water droplets, aerosols, direct contact and self-inoculation. Among avian species it can also spread via faecal-faecal, faecal-oral or faecal-respiratory tract transmission. Contracting the virus directly from water is also possible (Wang and Fish, 2017; Krammer et al., 2018).

First, the virus binds the host cell via HA, which recognizes receptors ending with sialic acid (SA). While human IAVs bind α2,6-linked SA receptors, present in epithelial cells of human upper respiratory tract, avian influenza viruses (AIVs) prefer α2,3-linked SA located in lower human respiratory tract, as well as in respiratory and intestinal tracts of birds. In addition to that, some avian viruses have shown the affinity for both α2,3 and α2,6-linked SA.

The fact that avian viruses tend to infect lower respiratory tract in humans may partially explain the severity of the infection (van Riel et al., 2013; Chen et al., 2017).

After receptor binding, the virus is then taken inside of the cell via endocytosis. The environment within the endosome is then acidified, which leads to conformational changes of HA and opening of M2 ion channel. This allows for influx of H^+, acidifying the inside of the virion. which leads to the fusion of viral and endosomal membranes and uncoating of the virion. Released RNPs are then moved into the nucleus via interactions with importins. Viral components are then transcribed by viral RNA polymerase complex. However the cap is added using host machinery. Processed transcripts are then translated. New virions can then bud off the membrane due to NA activity, which cleaves off SA residues, and can continue infecting neighbouring cells (Hsu, 2018; Han, Jeong & Jang; 2019).

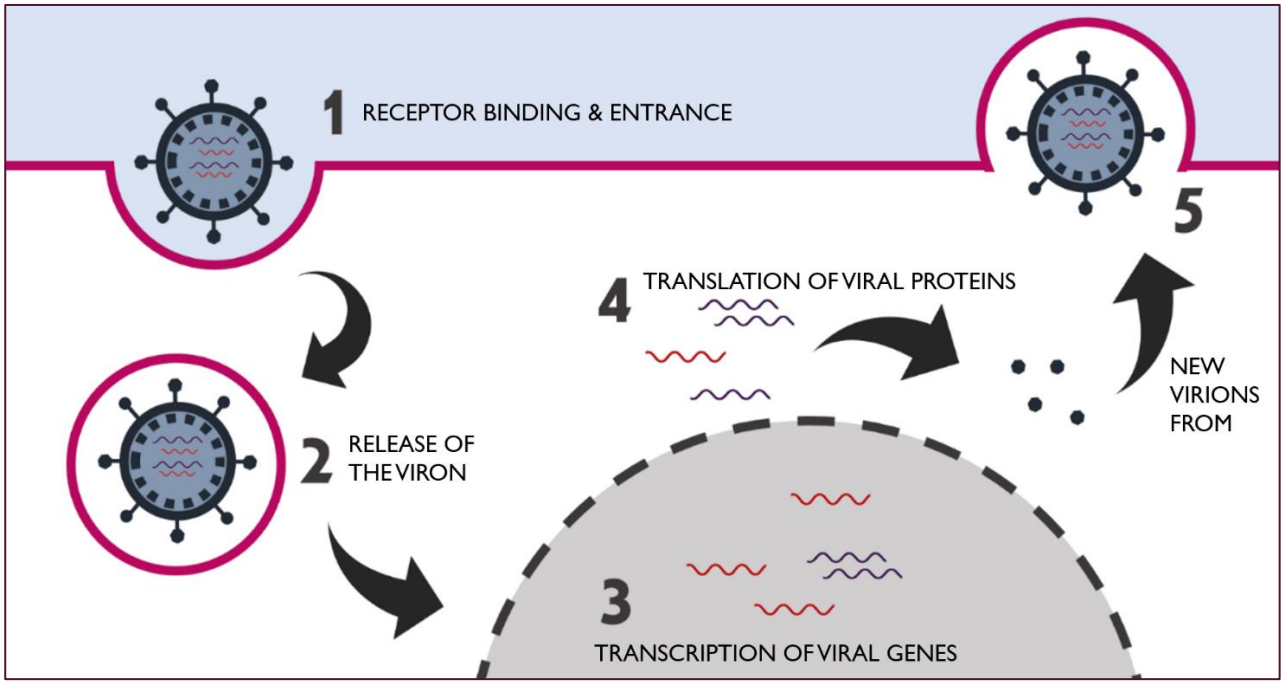


Figure 2. Replication of IAV. (1) Entry of the virus via endocytosis. (2) Release of the virus from endosome followed by uncoating of the virion. (3) Released RNPs translocate to the nucleus. Viral genes are transcribed by viral polymerase. (4) Translation of viral mRNA in the cytoplasm using host cell machinery (5) Newly formed viruses bud off the membrane, which becomes their viral envelope.

2.3. Pandemic potential of IAVs

IAVs are challenging for the immune system because their genome constantly changes. This may be partially attributed to the fact that RNA polymerase is more error prone than cellular DNA polymerase. In addition to that, IAVs have segmented genomes, which may lead to their reassortment (Krammer et al., 2018).

Antigenic drift, caused by the gradual accumulation of mutations in surface antigens, particularly HA, is responsible for seasonal flu epidemics (Fig. 3). Because of these mutations, antibodies produced during previous infections can no longer recognize the viral antigens and virus is able to successfully replicate (Mostafa et al., 2018). These biannual outbreaks, usually during winter in northern and southern hemispheres, are responsible for deaths of estimated 500 000 people worldwide (Girard et al., 2005). The groups at particular risk of infection are pregnant women, children younger than one year old and people over the age of 65 (Krammer et al., 2018). Currently circulating seasonal flu viruses are: A/H3N2 and A/H1N1, responsible for 73% of flu cases in 2018-2019 in Europe, whereas 27% of infections were caused by IBV subtype, mostly attributed to B/Victoria (European Centre for Disease Prevention and Control, 2020).

To limit the scope of these outbreaks, each season a new vaccine is created based on the epidemic strains of the previous season, usually consisting of HA and NA proteins. The composition of the vaccines is based on the surveillance done by many centres around the world, which characterize circulating strains and attempt to predict which ones will be most prevalent given year (Krammer et al., 2018).

Sometimes humans can also be infected by avian strains, but these cases show limited human-to-human transmission. The people affected are usually poultry workers. The viruses find their way from wild birds into chickens via domesticated ducks and geese. This makes so called *backyard farming*, where different avian species are placed together, particularly prone to becoming the source of such outbreaks. The adaptation of avian viruses to human host depends on many factors, for example sensitivity of viral polymerase to temperature, since avian polymerase tends to work better at lower temperatures than human one (Krammer et al., 2018). It was previously shown that introduction of only three mutations to avian H5N1 subtype is enough for it to establish transmission between ferrets. This is concerning, because

ferrets are thought to be good models for studying influenza infections in humans (Herfst et al., 2012).

In addition to epidemics, sometimes a novel virus can emerge, causing a pandemic. This may be a result of antigenic shift (Fig. 3). Since influenza viruses are continuously circulating among their hosts, two different viruses can infect the same cell and exchange their genetic segments. This may grant a virus the ability to cross the species barrier or increase its pathogenicity. Zoonotic IAVs possess a particular advantage, since they are entirely new to the human immune system and can cause a more severe infection (Widdowson, Bresee & Jernigan, 2017). This is additionally emphasized by the fact that all of the pandemics in the last century originated from AIVs (Fig. 4) (Kawaoka & Horimoto, 2005; Mostafa et al., 2018).

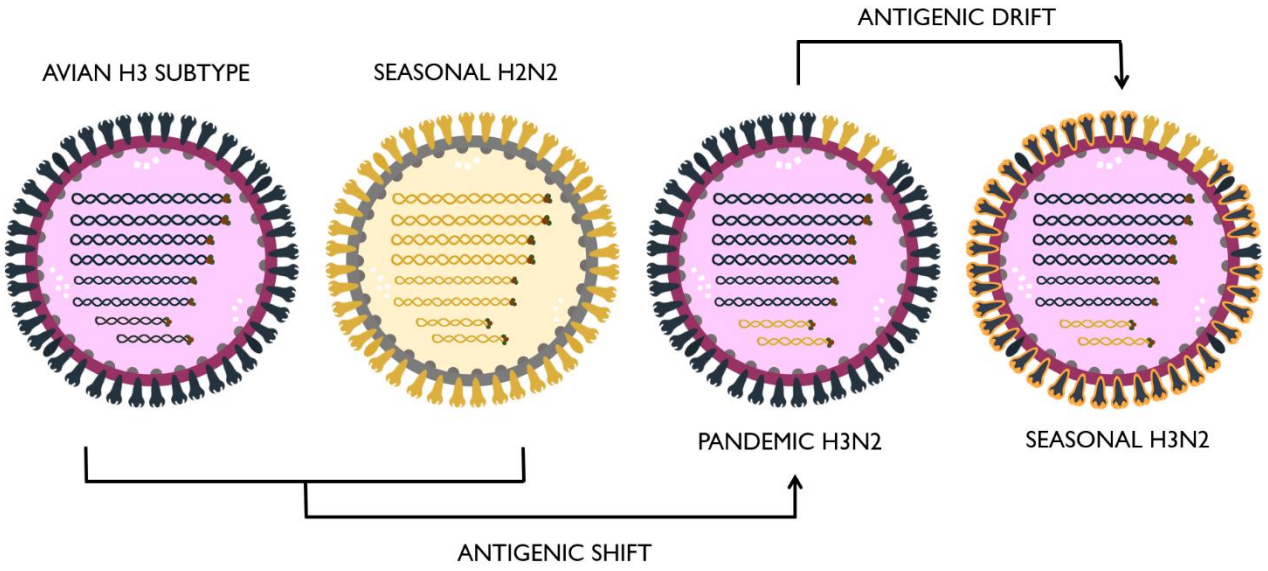


Figure 3. Difference between antigenic drift and antigenic shift. Antigenic shift may occur due to simultaneous infection of two different strains, for example one avian and one human, which may lead to the exchange of vRNA segments. The new reassorted virus can acquire new traits relatively quickly. Antigenic drift is a result of a slower process, including gradual acquisition of mutations in surface receptors (Krammer et al., 2018; modified).

The most deadly of recent pandemic, Spanish flu, was most likely caused by H1N1 antigenic subtype. It lasted from 1918 to 1919 and it is estimated that 50-100 million people, out of around 500 million infected, had died. Additionally, unlike other influenza outbreaks, which exhibit highest death toll among the youngest and the oldest, *Spanish flu* shown additional peak of deaths in adults between 20-40 years of age (Kilbourne, 2006; Taubenberger, Kash & Morens, 2019).

The following pandemic, called Asian flu, occurred in 1957. It was caused by H2N2 subtype, responsible for 1-2 million deaths worldwide. The virus emerged due to reassortment between H1N1 from 1918 with an AIV strain. Around a decade later, in 1968 another global outbreak originated in Southeast Asia: Hong Kong flu. The subtype responsible for it was H3N2. The virus was an effect of reassortment of H2N2 virus with avian H3-type. Around 700 000 fatal cases have been reported.

In 1977 H1N1 strain has reappeared, causing an epidemic, called Russian flu, also referred to as an age-restricted pandemic (Kilbourne, 2006). The virus was infecting mostly people 25 years old or younger, with 50% mortality rate among children. It is thought to be a result of lack of immunity against H1 subtype in people born after 1957, when H1N1 was replaced by H2N2 virus (Kilbourne, 2006; Mostafa et al., 2018).

The most recent outbreak, swine flu, classified by some sources as a pseudo-pandemic (Kilbourne, 2006), took place in 2009 – 2010. It was caused by H1N1 subtype that emerged due to triple reassortment in swine host: it contained several segments from human, swine and avian influenza strains. Over 360 000 people have died during that pandemic. However, this virus is now circulating as the seasonal H1N1 among humans after adapting to human host, and thus it has killed more people after that pandemic season (Kilbourne, 2006; WHO, 2016).

Both pandemics and seasonal epidemics have great social and economic impact. It is not a surprise that there are attempts to predict and prepare for potential new outbreaks. The strategy includes, among others, monitoring currently circulating AIVs, which in the future may give a rise to a new pandemic strain, as well as attempting to develop pre-pandemic vaccines (WHO, 2017).

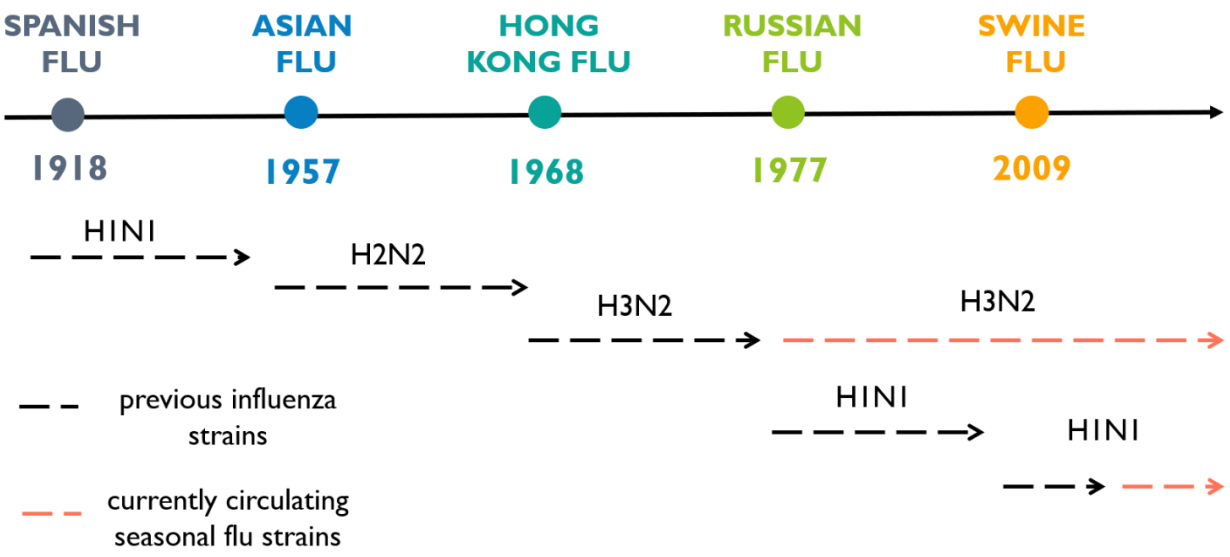


Figure 4. Timeline of influenza pandemics and IAVs circulation**.** Spanish flu was caused by H1N1 virus. Then the subtyped was replaced by H2N2 which caused Asian flu. This was followed by replacement of H2N2 by H3N2 subtype (Hong Kong flu). Then H3N2 adapted and started circulating as seasonal strain. In 1977 H1N1 unexpectedly reappeared causing Russian flu. In 2009 a different H1N1 strain emerged due to reassortment between human, avian and swine strains (swine flu), replacing the previous one. The new H1N1 subtype seemed to undergo antigenic change and is now circulating along with H3N2 as a seasonal flu strain.

2.4. Avian Influenza Viruses

AIVs are classified into low pathogenic (LP) and highly pathogenic (HP) strains based on their pathogenicity in chickens. Both HP and LP viruses have previously caused human infections (Table 1). Although this division does not reflect the severity of the infection in humans, HPAIVs tend to be more pathogenic also in humans and cause more acute symptoms (Hsu, 2018; Mostafa et al., 2018).

AIVs, especially those circulating in poultry, have previously caused outbreaks in humans. The reason might be the fact that viruses isolated in domesticated birds seem to evolve faster than in wild species, possibly due to continuous adaptation to new hosts (Mostafa et al., 2018). Several AIV strains are currently being closely examined, since they have shown sporadic animal to human transmissions. They do not currently pose a threat, but further mutations and reassertions may lead to them becoming pandemic.

2.4.1. H5N1

An example of such strain is H5N1 HP virus. The subtype is very diverse: viruses are classified into first, second, third and fourth order clades and then into sub-clades. Currently only 6 clades are circulating in poultry in Egypt and Southeast Asia (Marinova-Petkova et al., 2014). Overall, in the span of 15 years (2003–2018) H5N1 has infected 860 people worldwide with a 53% mortality rate (WHO, 2018a). It was first shown to cross species barrier in Hong Kong in 1997 during an outbreak in poultry when 18 people were infected (Lin et al., 2000; Kawaoka & Horimoto, 2005; Parry, 2013). Genetic analysis has shown that several RNA segments were acquired from H9N2 virus. The same segments were isolated two years later from two patients, which implies they my support poultry-to-human transmission (Lin et al., 2000).

The same virus re-emerged in another outbreak in poultry in 2004. The virus spread from Vietnam to China, Indonesia, Thailand, and Cambodia (WHO, 2014). It was shown that the virus was slowly increasing its ability to infect mammals (Chen et al., 2004). Although the possibility of pandemic is currently low, the possible severity of it is the main source of concern.

2.4.2. H7N9

Another important subtype is H7N9. This LPAIV strain was first isolated in three patients in 2013 in eastern China experiencing symptoms of severe pneumonia (Parry, 2013). The virus has shown occasional interspecies transmission, as well as few cases of non-sustained human-to-human transmission (van Riel et al., 2013; Peiris et al., 2016; Mostafa et al., 2018). Unlike other AIV strains, H7N9 is able to attach to both lower and most importantly, upper respiratory tract (van Riel et al., 2013). In addition to that, an emerging HPAIV H7N9 strain was discovered four years later. The acquired mutations increased the activity of viral polymerase, and thus replication (Yamayoshi et al., 2018). However, it is not clear if these mutations significantly affect the virulence (Mostafa et al., 2018).

2.4.3. H7N7

H7N7 HPAIV has been previously reported to infect people, with the most human cases occurring during an outbreak in poultry in 2003 in Netherlands. The number of infections was

higher than expected, reaching 89 people. Most people experienced only mild symptoms, but one fatal case was reported (Van Kolfschooten, 2003; Koopmans et al., 2004). In 2013 a LPAIV H7N7 virus containing genes from H9N2 subtype was discovered in chickens in China. There were no human infections, but the virus was experimentally shown to infect mammals (Lam et al., 2013).

2.4.4. H9N2

H9N2 LPAIV, despite usually not causing any clinical symptoms, has been closely monitored. The strain may pose a risk due to its relatively high incidence rate of zoonotic transmission and its prevalence among poultry in Europe and Asia (Schrauwen and Fouchier, 2014). Additionally, the presence of antibodies among poultry workers in affected region suggests that most cases are quite mild and often go undetected (Li et al., 2017).

All previously discussed strains (H5N1, H7N9, H7N7) have acquired some of the genes from H9N2. It is thought they play a role in an increased transmission efficacy between species (Mostafa et al., 2018). Moreover, H9N2 can also infect pigs, which are said to be an intermediate between avian and human infections (Sun et al., 2020).

Table 1. Chosen AIVs showing pandemic potential

Subtype	Summary
H5N1	53% mortality rate, severe symptoms
H7N9	38% mortality rate, severe symptoms, multiple human outbreaks
H7N7	mild symptoms, 1 fatal case, few human outbreaks
H9N2	low mortality rate, high risk of zoonotic transmission

Monitoring poultry-to-human infections has shown that AIVs are constantly evolving. Mixing genes from different strains may lead to increased pathogenicity and eventually more effective spreading potential. Understanding which factors contribute to infection, replication and interspecies transmission is important for better pandemic preparedness.

2.5. Immune antiviral response

In order to prevent the entry and replication of a pathogen, there are two lines of defence: innate immunity and adaptive immunity. Innate immunity is unspecific, as it

responds to elements shared by many pathogens, such as double-stranded DNA or components of a bacterial wall. It consists of physical barriers, such as skin and mucus, as well as phagocytosis, cytokines and other proteins. Adaptive immunity is more specific and can be divided into cell-mediated immunity and antibody-mediated immunity. Both innate and adaptive immunity have a role in supressing viral infections (Krammer et al., 2018).

2.5.1. RIG-I pathway

When pathogen gets through physical barriers, first step leading to the immune response is detection of its entry. This can happen through pattern recognition receptors (PRRs), which recognize pathogen associated molecular patterns (PAMPs). PPRs can be either membrane bound or cytoplasmic. The most important pathway in influenza virus detection is retinoic acid-inducible gene I (RIG-I) pathway (Fig. 5), which leads to the production of interferons (IFNs) (Pichlmair et al., 2006).

Signalling begins when RIG-I is activated by binding PAMPs, such 5' triphosphorylated end of double-stranded RNA (dsRNA). The signal is then transduced via adaptor protein MAVS to inhibitor of nuclear factor kappa B (IκB) kinase α (IKKα), IKKβ and IKKγ complex, which can phosphorylate the IκB and one of the subunits of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factor. This leads to disassociation of IκB from NF-κB and its degradation in a proteasome. Released and phosphorylated NF-κB can then translocate to the nucleus via importins and bind NF-κB binding site in the IFN promoter. Another kinase complex that is activated by MAVS, IKKε and TANK-binding kinase 1 (TBK1), can phosphorylate interferon regulatory factor 3 (IRF3) in order for it to create a homodimer and bind interferon-sensitive response elements (ISRE) within IFN gene promoter. IRF3, which is expressed constitutively, can also heterodimerize with IRF7, present in the cell in a low amount but expressed strongly in response to stimulation by IFNs, thus creating positive feedback. All of the above virus-induced signalling pathways activate IFN expression, which helps the cell fight the infection (Jiang et al., 2015).

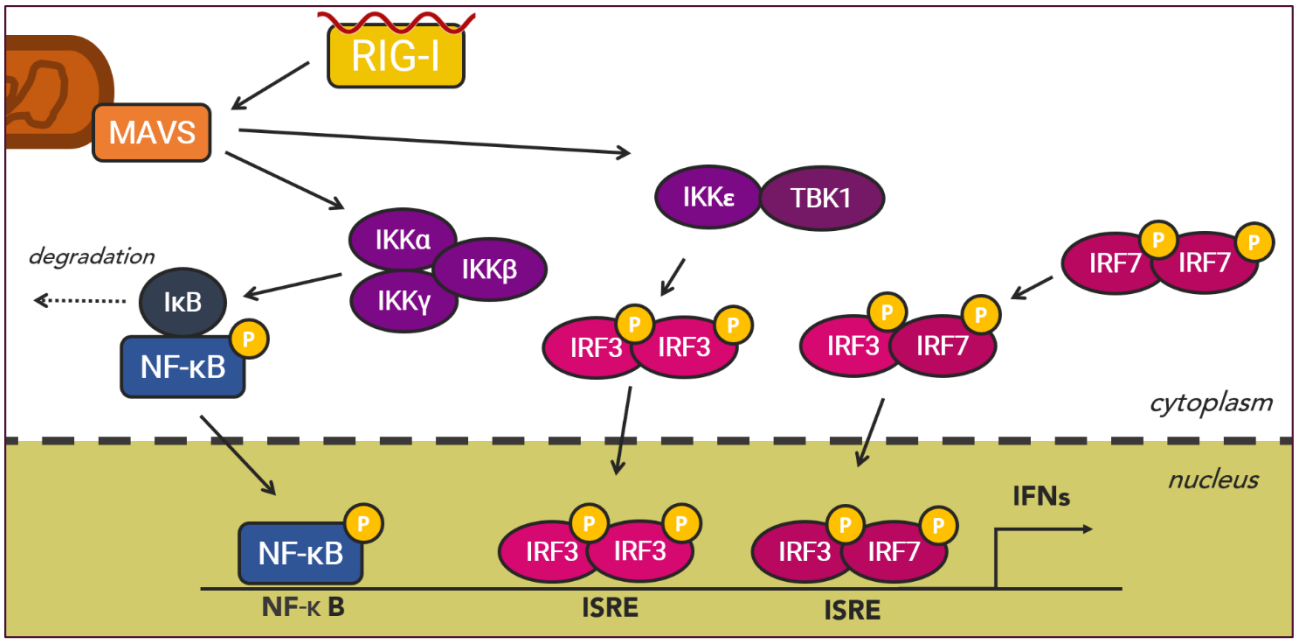


Figure 5. RIG-I pathway. Viral RNA is bound by RIG-I protein which triggers signalling cascade via MAVS and the above kinases (IKKα, IKKβ, IKKγ, IKKε and TBK1) leading to phosphorylation of several transcription factors. These factors then translocate to the nucleus and bind regulatory elements within IFN gene promoter (Jiang et al., 2015; modified).

2.5.2. Interferon functions

Interferons are a group of cytokines with versatile functions in overcoming an infection. They can be divided into three classes based on their receptors: type I, type II and type III. Type I interferons include several IFN-α subtypes, IFN-β, IFN-ε, IFN-κ and IFN-ω. Type II interferon only consist of IFN-γ and type III IFN of IFN-λ1-4. Type I and III are produced by epithelial cells, macrophages and dendritic cells upon the infection in order to inhibit viral replication. Then the release of inflammatory cytokines attracts more immune cells, such as natural killer cells. Interferons can greatly limit the infection, but excessive cytokine production may lead to lung damage (Krammer et al., 2018). Of type I and type III IFNs, both IFN-β and IFN-λ1 promoters contain IRF and NF-κB binding sites (Österlund et al., 2007; Pietilä et al., 2007). Type II IFNs differ from type I and III IFNs, since they are mainly produced by immune cells. In addition to that, they are stimulated by other IFNs and interleukins, only in lesser extent by PAMPs (Castro et al., 2018).

2.5.3. Interferon signalling

Despite might having evolved separately, IFNs use a similar signalling pathway (Fig. 6). The binding of these IFNs to their own type of receptors, IFNAR for type I and IFNLR for type III IFNs, leads to the phosphorylation of the janus kinase (JAK) -mediated activation of signal transducer and activator of transcription 1 and 2 (STAT1/2) proteins, and this allows IRF9 binding. The formed interferon stimulated gene factor 3 complex then translocates to the nucleus and binds to ISRE elements triggering the transcription of interferon stimulate genes (ISGs) (Platanias, 2005).

Upregulated expression of IFNs regulates the state of infected and neighbouring cells via autocrine and paracrine signalling. It is manifested mainly by modulating immune response, cell growth and metabolism (Wang and Fish, 2017).

It has been shown, that different IAVs differ significantly when it comes to inducing IFN-β expression (Han, Jeong & Jang, 2019). H7N9 strain can avoid host-cell recognition very well and IFN induction is lower than in cells with seasonal flu virus infection. On the contrary, H5N1 virus causes so called cytokine storm, yet it is able to replicate very effectively (Arilahti et al., 2014).

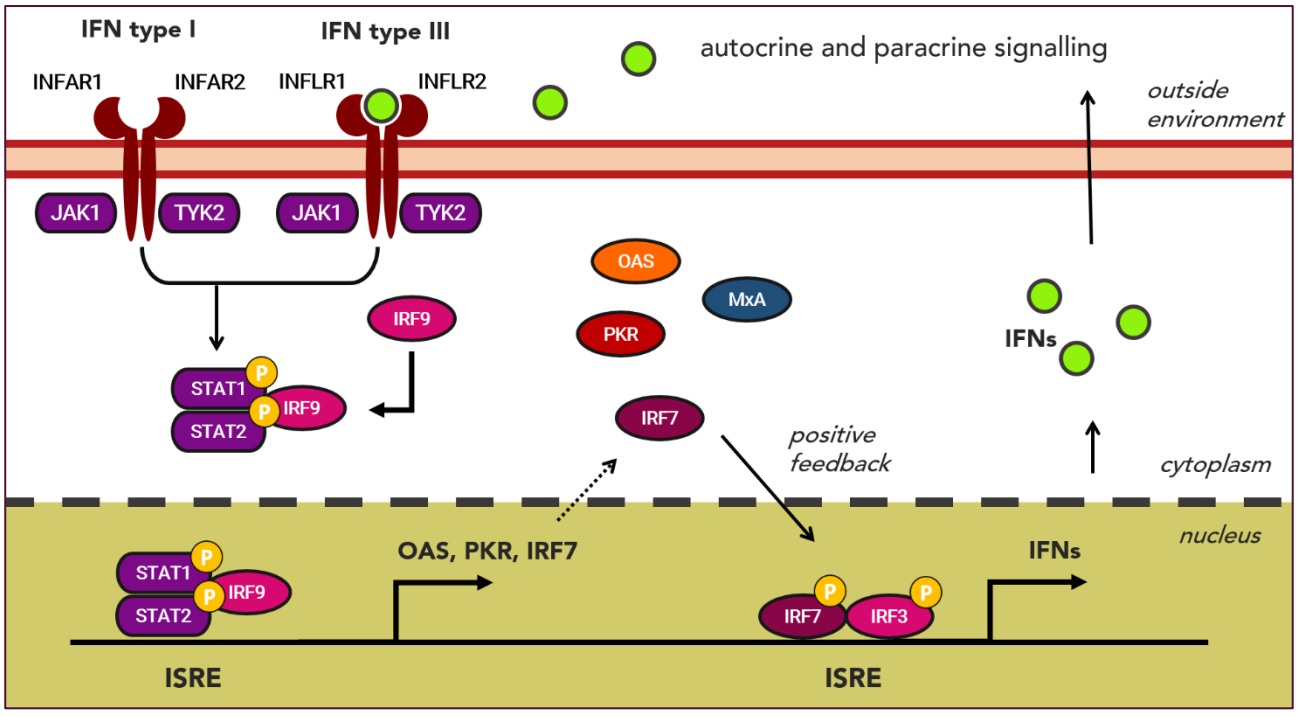


Figure 6. Interferon signalling pathway and ISGs expression. Upon IFN binding both IFN type I and III trigger signalling cascade that leads to the phosphorylation of STAT1/2 proteins. A

heterotrimer with IRF9 is formed and translocated to nucleus, where it binds to ISRE within ISGs promoters. The binding allows for expression of various ISGs, such as OAS, PKR, IRF7 and MxA, which creates antiviral state in infected and neighbouring cells. IRF7 also additionally upregulates IFN genes expression and acts as a positive regulator of transcription (Julkunen et al., 2001; modified).

2.5.4. Interferon stimulated genes

An important role of IFNs during viral infection is increasing the expression of various ISGs, such as myxovirus resistance gene A (MxA), IRF7, 2'-5' oligo (A) synthetase (OAS) and RNA-dependent protein kinase (PKR), which further induce antiviral state in infected or neighbouring cells.

MxA is an important protein in influenza infection in humans is. It is a cytosolic GTPase induced only by IFNs and STAT signalling. Infection of the virus alone is not enough to activate its expression. Its main role is inhibition of viral protein synthesis (Pavlovic, Haller & Staeheli, 1992; Haller & Kochs, 2011). In addition to that, MxA modified to localize in nucleus was shown to inhibit viral transcription via interactions with NP (Turan et al., 2004). The sensitivity to its activity differs between IAV strains: avian strains seem to be more affected than human viruses (Haller & Kochs, 2011).

IRF7 is a major regulator of IFN gene expression. Small amounts of IRF7 are normally present in cytoplasm in an inactive form. However, in early stages of the infection PRRs phosphorylate IRF7, and thus activating it. This allows for its translocation to nucleus and activation of IFN promoter. IFNs in turn increase the expression of IRF7, creating a positive feedback loop (Drappier & Michiels, 2015).

OAS is a protein activated by dsRNA, responsible for synthesis of short 2'5' oligonucleotides that act as activator for RNase L. RNase L dimerized and cleaves both viral and cellular single-stranded RNA. The former directly prohibits replication of viral genome, the latter leads to apoptosis of infected cell, which limits the spread of the virus (Drappier & Michiels, 2015). In addition to that, cleaved segments of RNA may further activate RIG-I (Nogales et al., 2018).

PKR is constitutively expressed in cells, but can be further expressed and activated in two ways: through binding of dsRNA or by protein activator of the interferon-induced PKR (PACT). PKR is then able to phosphorylate cellular proteins, including eukaryotic initiation

factor 2, which inhibits protein synthesis of both cellular and viral proteins (Li et al., 2006; Klemm et al., 2018; Nogales et al., 2018).

Although the ISGs described above are the most relevant regarding influenza infection, there are many other including ISG15, which acts through ISGylation of proteins relevant for viral replication (Perng & Lenschow, 2018), or interferon induced transmembrane protein 3, which prohibits fusion of host and viral membranes at the first step of the infection (Fig. 2) (Nogales et al., 2018).

RIG-I pathway, IFN expression and signalling, as well as ISGs expression are the most important factors leading to an effective antiviral immune response. However, those mechanisms are not always enough to prohibit the spread of the virus.

2.6. Viral immune evasion

In order to replicate successfully, viruses had to evolve mechanisms to overcome host defences. They include, among others, regulation of IFNs and ISGs expression. During IAV infection, these functions are mostly mediated by NS1 protein (Hale et al.,2008).

2.6.1. Structure and synthesis of NS1

NS1 is a protein encoded by the eighth, shortest RNA segment of the influenza virus, along with NEP. Both NEP and NS1 transcripts are a result of splicing. Their sequences are not independent: they share ~56 nucleotides at the 5'end of the transcript (Lamb & Lai, 1980). NS1 is expressed at high level in infected cells. Its synthesis seems to be partially autoregulated: it has been shown that NS1 can inhibit the splicing of the mRNA leading to the downregulation of NEP expression. This explains the considerably lower amount of NEP as compared to the levels of NS1 found in infected cells (Garaigorta & Ortin, 2007). Even though it is considered to be a non-structural protein, it is found in small amounts in the virion (Hutchinson et al., 2014).

NS1 has a molecular weight of around 26 kDa and consists of 215-237 amino acids (Han, Jeong & Jang, 2019). It comprises the RNA-binding domain (RBD) capable of binding RNA independently of the sequence, the linker domain (LD), the effector domain (ED) responsible for interactions with host cell proteins and stabilizing RBD and the C-terminal tail (CTT). A nuclear localization signal (NLS) is encoded within RBS. Most IAVs also contain second NLS within CTT and nuclear export signal (NES) within the effector domain (Fig. 7). NS1 protein

is present in the cell as a homodimer. Both RBD and ED contribute to the dimerization (Hale et al., 2008).

NS1 can be found mainly in the nucleus, but it is still present in a significant amount in cytoplasm, especially in later stages of the infection. Its localization in the cytoplasm may be mediated by three different mechanism: initial accumulation of transcripts in the cytoplasm along with masking of NLS, unmasking of NES or by competition between NLS and NES (Hale et al., 2008). The distribution of NS1 between cytoplasm and nucleus may be a strain-specific trait (Wang and Fish, 2017).

There is variation in the amino-acid chains of the protein between different IAV strains. For example, H3N2 shares only around 67% of the sequence of NS1 of H5N1. Differences in the effector domain are said to be responsible for changes in the functionality of NS1 (Han, Jeong & Jang, 2019). Another factors adding to variations between strains is post-translational modifications of NS1, such as phosphorylation (Hale et al., 2008).

NS1 is a multifunctional protein responsible, among others, for interfering the host antiviral response and determining viral pathogenicity (Fig. 8).

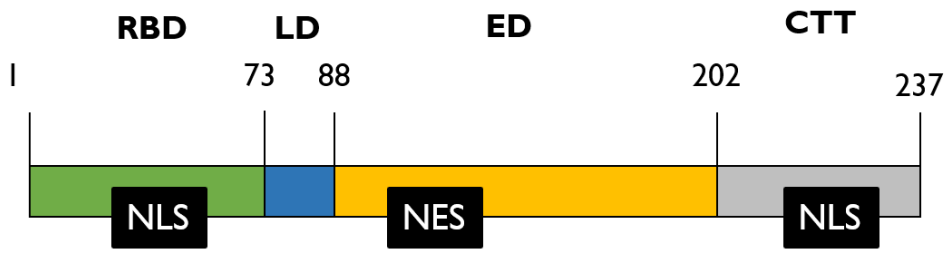


Figure. 7 Schematic structure of NS1**.** NS1 protein consists of ~237 amino acids. It comprises RNA-binding domain (RBD), linker domain (LD), effector domain (ED) and C-terminal domain (CTT). Most of the strains contain two nuclear localization signal (NLS) sequences and one nuclear export signal (NES) sequence (Nogales et al., 2018; modified).

2.6.2. Inhibition of interferon gene expression

The main role of NS1 that enables escaping immune evasion is avoiding detection and thus inhibition of an interferon response. This can be done at various steps of the RIG-I pathway. It was shown that NS1 can compete with RIG-I for 5'-triphosphate vRNA binding. RIG-I pathway induction may also be limited by binding of NS1 to tripartite motif-containing

protein 25 (TRIM25), which inhibits ubiquitination, and thus the activation of RIG-I (Gack et al., 2009). Another mechanism of NS1 in suppressing IFN signalling includes preventing transcription factors from entering the nucleus, either by binding the IκB and protecting it from degradation, or by blocking the phosphorylation of IRF3 (Klemm et al., 2018).

In addition to that, NS1 can inhibit IFN signalling via inhibition of host mRNA production and processing. It is achieved via cleavage and polyadenylation specificity factor 30 (CPSF30) and poly(A)-binding protein II (PAB II) binding, which take part in adding poly(A) tail to transcripts (Keller et al., 1991; Hsu, 2018).

2.6.3. Inhibition of ISGs

Another way NS1 can mitigate the result of IFN expression is interfering with ISGs. Inhibition of viral detection via RIG-I and STAT-signalling alone indirectly affects MxA and IFR7 activity, since these proteins heavily rely on PPRs and IFNs. In case of ISGs such as OAS or PKR, which are activated by binding of vRNAs, NS1 prevents their activation via sequestration of dsRNA though its RBD. In addition to that, PKR can also be inhibited directly through NS1 binding (Li et al., 2006; Nogales et al., 2018).

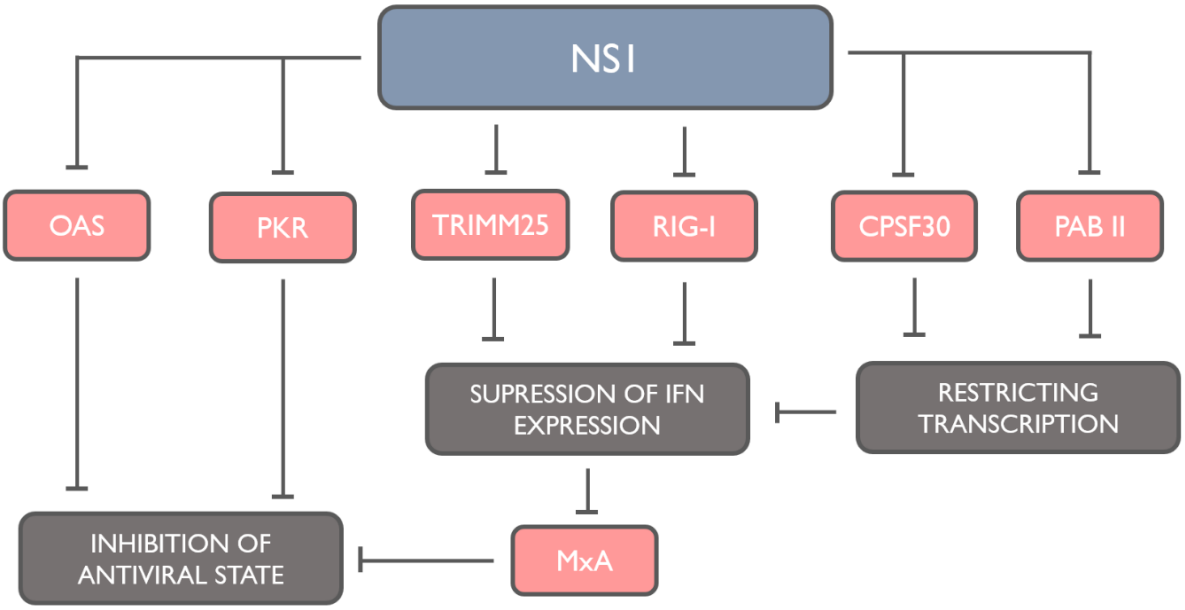


Figure 8. Summary of chosen functions of NS1 protein. NS1 can promote viral replication via several mechanisms. They include suppression of IFN genes expression through competing for vRNAs with RIG-I and interactions with TRIM25, as well as inhibition of antiviral state

through interference with ISGs and restricting transcription through CPSF30 and PAB II binding (Han, Jeong & Jang, 2019; modified)

2.6.4. Other NS1 functions

NS1 can benefit the virus in many other ways, one of them being the activation of phosphoinositide 3-kinase (Pi3K) pathway, which supresses apoptosis before replication is complete. Additionally, Pi3K can act through IRF3 as an alternative path for IFNs induction. NS1 is also capable of DExD-box helicase 21 binding, which when unbound prohibits it from interfering with assembly of viral polymerase complex. NS1 has also been shown to assist in the preferential translation of viral mRNAs via the recruitment of eukaryotic translation initiation factor 4GI to their 5'UTR (Klemm et al., 2018; Han, Jeong & Jang, 2019).

The plurality of mechanisms through which NS1 can modulate cell's immune response against infection highlights its importance in determining efficiency of viral replication and pathogenicity.

2.6.5. NS1 and efforts to control influenza outbreaks

Given the importance of NS1 in viral pathogenicity, the protein seems to be an obvious target for the creation of live attenuated vaccines. There have been several studies using viruses lacking NS1 gene as potential vaccines. An example can be a vaccine against H1N1 virus that is currently in phase I clinical trials. It was shown that IgA antibodies were able to neutralize not only H1N1 subtype, but also were effective for H5N1 and H3N2 viruses (Wacheck et al., 2010; Morokutti, Muster & Ferko, 2014) Another vaccine which is in phase I/II clinical trial aimed at H1N1, H3N2 and one of IBV seasonal strains has been shown to be safe for use (Mössler et al., 2013).

There are also vaccines developed for chickens, in order to limit the prevalence of IAVs in poultry and thus decreasing the risk of a pandemic arising from an avian strain. An example is a vaccine against H9N2 virus with a truncated NS1 gene. This vaccine has shown greater immunization when compared to an inactivated one (Chen et al., 2017).

Currently used antivirals are targeting either M2 ion channel or NA. There are usually used only for patients experiencing severe symptoms, but in case of the pandemic they would be crucial to manage the outbreak (Krammer et al., 2018). However, there are no alternatives in case of resistance towards the treatment. This creates a need for finding new drug targets.

NS1 is one of the proteins of interest due to its key role in successful viral replication. The approaches include designing short interfering RNAs, anti-NS1 antibodies and other small molecules targeting various NS1 interactions, particularly RNA binding. Unfortunately, these studies had very little success (Krammer et al., 2018; Rosário-Ferreira et al., 2020).

3. Aims

The aim of this Master's project was to assess the changes in activation of type I and III interferon gene promoters in presence of NS1 proteins originating from five different avian influenza strains and one seasonal influenza strain.

4. Materials and methods

4.1. Cell cultures and virus infection

Human embryonic kidney epithelial cell line (HEK293) was maintained in (EAGLE-MEM) (Sigma-Aldrich) supplemented with antibiotics (penicillin and streptomycin), L-glutamine, HEPES and 10% fetal bovine serum and split every third day or when the confluency was approx. 70%. For transfection experiments cells were plated in 24-well plates using medium supplemented with only 2% fetal bovine serum around 15-20h before the transfection. Preferred confluency for the transfection was 80-90%. The parameters of the incubator were 5% carbon dioxide concentration with humidity and temperature of 37°C.

4.2. Plasmids

For IFN promoter-reporter constructs IFN-β and IFN-λ1 promoter sequences were cloned into the pGL3-basic vector expressing the firefly luciferase (FFluc) (Promega) as described previously (Österlund et al., 2007). IFN-β promoter sequence was inserted into NheI restriction sites and IFN-λ1 between MluI and XhoI (Fig. 9a). Renilla-luc (Rluc) plasmid was creating analogically.

Constructs containing different influenza virus NS1 genes with a N-terminal flag-tag sequence were cloned in the pcDNA3.1 plasmid (Julkunen et al., 2001). The NS1 gene was driven by a T7 constitutive promoter (Fig. 9b). The sequences came from seasonal flu IAV strains: A/Beijing/353/1989 (H3N2), and 5 different AIV strains: A/HK/156/1997 (H5N1/97),

A/VN/1203/2004 (H5N1/04), A/NL/219/2003 (H7N7), A/Anhui/1/2013 (H7N9) and A/HK/1073/1999 (H9N2).

PmKate2-NS1 H5N1 A/HK/156/97 was constructed by cloning the NS1 gene sequence from H5N1 A/HK/156/97 fused to the mKate2 N-terminus (Evrogen) into BamH1 site under a CMV promoter (Fig. 9c).

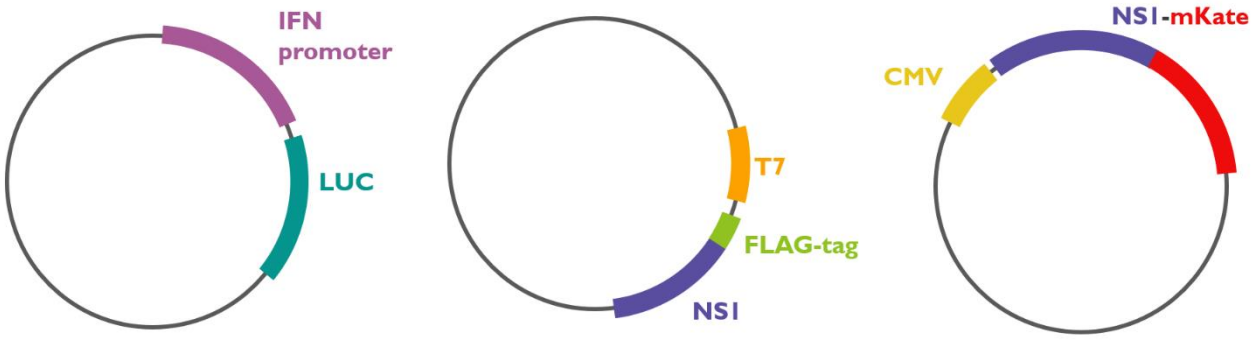


Figure 9. Simplified structure of plasmids used in the experiments. (A) Luciferase expression plasmid: IFN-β or IFN-λ1 promoter is driving a luciferase gene. (B) NS1 expression plasmid: flag-tagged NS1 gene driven by a T7 constitutive promoter. (C) NS1-mKate fluorescence fusion protein expression plasmid run by CMV constitutive promoter.

4.3. Transfection and IFN promoter induction

For the experiment cells were co-transfected with FFluc plasmids (100 ng/well), as well as a plasmid containing a gradient of NS1 (20 ng – 100 ng – 500 ng) from the 6 previously described influenza strains using TransIT®-2020 Transfection Reagent (Mirus). The amount of DNA was made up to 500 ng with an empty vector (pcDNA). *Rluc* plasmid (3 ng/well) was used as a control of transfection efficiency.

For live-cell imaging during experiment optimization flag-tagged NS1 expression plasmids were replaced with pmKate2-NS1 H5N1/97.

After 4 hours incubation, the cells were infected with Sendai virus (SV) (strain Cantell, originates from National Public Health Institute, Finland, the former THL and was cultured in embryonated chicken eggs and then stored at -70 °C) (Ronni et al., 1995). The virus was chosen because it is as strong inducer of IFNs. It also has less interfering properties than influenza viruses, which would produce additional NS1 during the infection. SV was diluted 1:100 in EAGLE-MEM. In order to analyse the IFN promoter-driven luciferase and the protein

expression of NS1 the cells were lysed in 1x passive lysis buffer (Promega) after 24 h incubation. Collected lysate was then separated for a luciferase assay and western blotting.

4.4. Protein expression analysis

4.4.1. Luciferase assay

A luciferase assay (Dual-Luciferase® Reporter Assay System, Promega) was performed to measure activation of interferon promoter using Victor multiplate reader (Wallac). The values were used to calculate fold induction of IFN gene promoter activation: FFluc counts per second (CPS) were divided by Rluc CPS. Negative control (ctrl -), the cell samples that were transfected with pcDNA and not infected with SV, was assigned the value 1. The fold induction for the rest of the samples was calculated in regard to that value. Positive control (ctrl +) comprised the cells that were transfected with pcDNA and infected with SV.

4.4.2. Western blotting

The cell lysate in passive lysis buffer (Promega) was mixed with 4x Laemmli buffer and boiled for denaturing the proteins before separated in 12% SDS-PAGE gel. After the electrophoresis proteins were transferred to Hybond-P polyvinylidene difuoride (PVDF) membranes (Amersham Biosciences). The membranes were blocked with 5% milk protein in PBS. The staining was done with primary anti-FLAG (Sigma F1804 M2, 1:1000 mouse dissolved in 5% BSA) and anti-GAPDH (Cell Signalling Tech, #2118 rabbit) antibodies at room temperature for 1 h. Secondary antibody staining was done with anti-mouse (for anti-FLAG staining) and anti-rabbit (for anti-GAPDH staining) HRP-conjugated antibodies (Dako) at room temperature for 1 h. Protein bands were visualized on HyperMax films using Pierce ECL plus system (Thermo Fisher Scientific).

5. Results

5.1. Optimization of the experiment

In order to assure the reliability of the data, several elements needed to be optimized. First, the appropriate amount of NS1-containing plasmid needed to be determined, so that the NS1 protein gradient is visible in blots and that the expression levels between different influenza

strains are relatively constant (Table 2). The gradient chosen for the experiment was 20 ng – 100 ng – 500 ng, since it created a visible differences between plasmids amounts.

Table 2. Optimization of NS1 expression plasmid amount

Gradient of NS1 expression plasmid	Result
50 ng – 200 ng – 500 ng	200 ng and 500 ng are too similar, difficult to distinguish
20 ng - 100 ng - 500 ng	20 ng band not visible; clear gradient between 200 ng and 500 ng

Secondly, the amount of Rluc-expressing plasmid, where Rluc is expressed at higher levels than IFN promoter-induced FFluc because it is driven by constantly high-active SV40 promoter, needed to be selected so that the background is not too strong regarding the IFN promoter-induced FFluc and the differences in IFN induction are visible. The amount chosen was 3 ng for Rluc and 100 ng for FFluc (Table 3).

Table 3. Optimization of Rluc- expressing plasmid amount

Rluc- expression plasmid	Result
10 ng – 100 ng	background too high, low induction fold
3 ng – 100 ng	lower background, higher induction fold

Lastly, the incubation time between the transfection and infection, as well as the dilution of the virus needed to be established. It was important to balance these factors in order to get sufficient transfection efficiency and expression of NS1 molecules, avoid killing too many cells, but at the same time to get detectable virus-induced IFN response. This was determined by live-cell imaging of mKate expression (Table 4). Optimal results were achieved using 1:100 dilution for the virus and 24 h incubation before the infection. However, when a new batch of cells was provided, cells with lower passage history displayed a satisfactory NS1 expression levels already after 4 h incubation. It is possible that initially 4 h incubation between transfection and infection was not enough to see mKate protein expression, given that transfection alone is already stressful for the cells. Viral infection may have caused too

many cells to die. Fresh batch of cells seemed to have a better vitality so 24 h incubation before the infection was not necessary.

Table 3. Assay to determine optimal parameters for IFN promoter induction

		Amount of time	
		4 h	24 h
Sufficient dilution of SV	1:100	mKate2-NS1 expressed in few cells	mKate2-NS1 expressed in most cells
	1:500	no IFN activation	no IFN activation

Additionally, in order to reduce variability between samples and replicates, a mix of pcDNA and NS1 plasmids for each strain was premade. Analogical mix was created for renilla and IFN promoter plasmids.

5.2. Expression of NS1 protein

To ensure the NS1 protein gradient is visible and that the expression level is comparable between strains, western blotting was performed (Fig. 10). The anti-FLAG antibodies were chosen, since anti-NS1 antibodies display different affinity between strains. This was followed with anti-GAPDH staining to control the equal loading between the wells.

With the selected plasmid concentrations most of the NS1 clones were expressed at the same efficiency. However, the NS1 clones originated from the H5N1/04 and H7N7 strains tended to be expressed at lower efficiency most of the times. The differences were not caused by incorrect loading, since GAPDH was expressed equally in all the wells. Regardless of the difference, the gradient of NS1 protein was still visible for all of the clones.

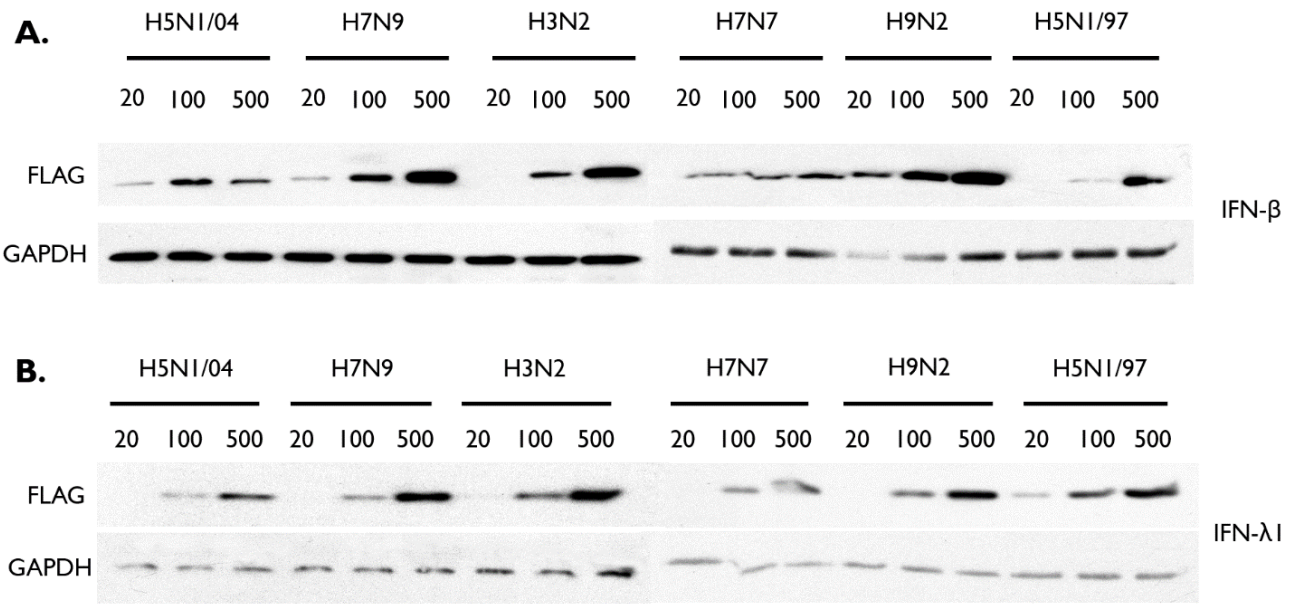


Figure 10 Expression of antiviral NS1 protein of H5N1/04 , H7N9, H3N2, H7N7, H9N2 and H5N1/97 in HEK293 cells. Cells were transfected with (A) IFN-β and (B) IFN-λ1 FFluc reporter plasmids along with a gradient (20 ng – 100 ng – 500 ng) of NS1 expression plasmids from chosen IAV strains. After 4 h incubation all samples were infected with SV. Cell lysates were collected 24 h after infection, analysed by Western blotting using anti-FLAG antibodies. GAPDH protein expression was analysed to control equal loading of the samples. A representative experiment out of 8 is shown.

5.3. Sendai-induced activation of IFN-β and IFN-λ1 promoters in the presence of different IAVs NS1 proteins

In order to stimulate IFN promoter activation, the cells were infected with SV. Following this, a luciferase assay was performed to assess the efficiency of different NS1 proteins in supressing IFN activation (Fig. 11).

The maximum observed fold induction of IFN-β promoter reached over 8-fold (Fig. 11a). It was suppressed the most by H3N2 (seasonal flu) NS1, closely followed by H7N9, H5N1/04 and H5N1/97. Regarding the luciferase results between 100 ng and 500 ng samples for H7N9, H5N1/04 and H5N1/97 strains, there is not a clear gradient in suppression of IFN activation although the expression of NS1 proteins in western blots show the difference between the amount of plasmids used (Fig. 10). However, the difference in IFN activation between these samples is minor and may imply that the strongest effect of these NS1 proteins are achieved

already in the 100 ng sample, so increasing its amount does not affect IFN promoter activation further. The lowest suppression in IFN-β activation was seen respectively with H7N7 and H9N2 strains. The gradient between all samples was visible and reflected protein expression seen in western blots.

The IFN-λ1 promoter showed greater induction by SV, which reached 24-fold (Fig. 11b). In all of the strains, IFN-λ1 activation corresponded to the NS1 gradient. As in the case of IFN-β, H3N2 was the most efficient in suppressing IFN-λ1 promoter. Very strong IFN promoter suppression was also observed for NS1 of H7N9 and H5N1/97. NS1 of H7N7 was not as efficient at suppressing IFN-λ1 promoter. IFN-λ1 activation in the presence of NS1 from H9N2 was rather high, ranging between a 9,5- and 5,4-fold change. The lowest suppression of IFN-λ1 promoter was observed for H5N1/04. However, this results for may be caused by the fact, that NS1 from H5N1/04 tended to be expressed at lower level (Fig. 10a).

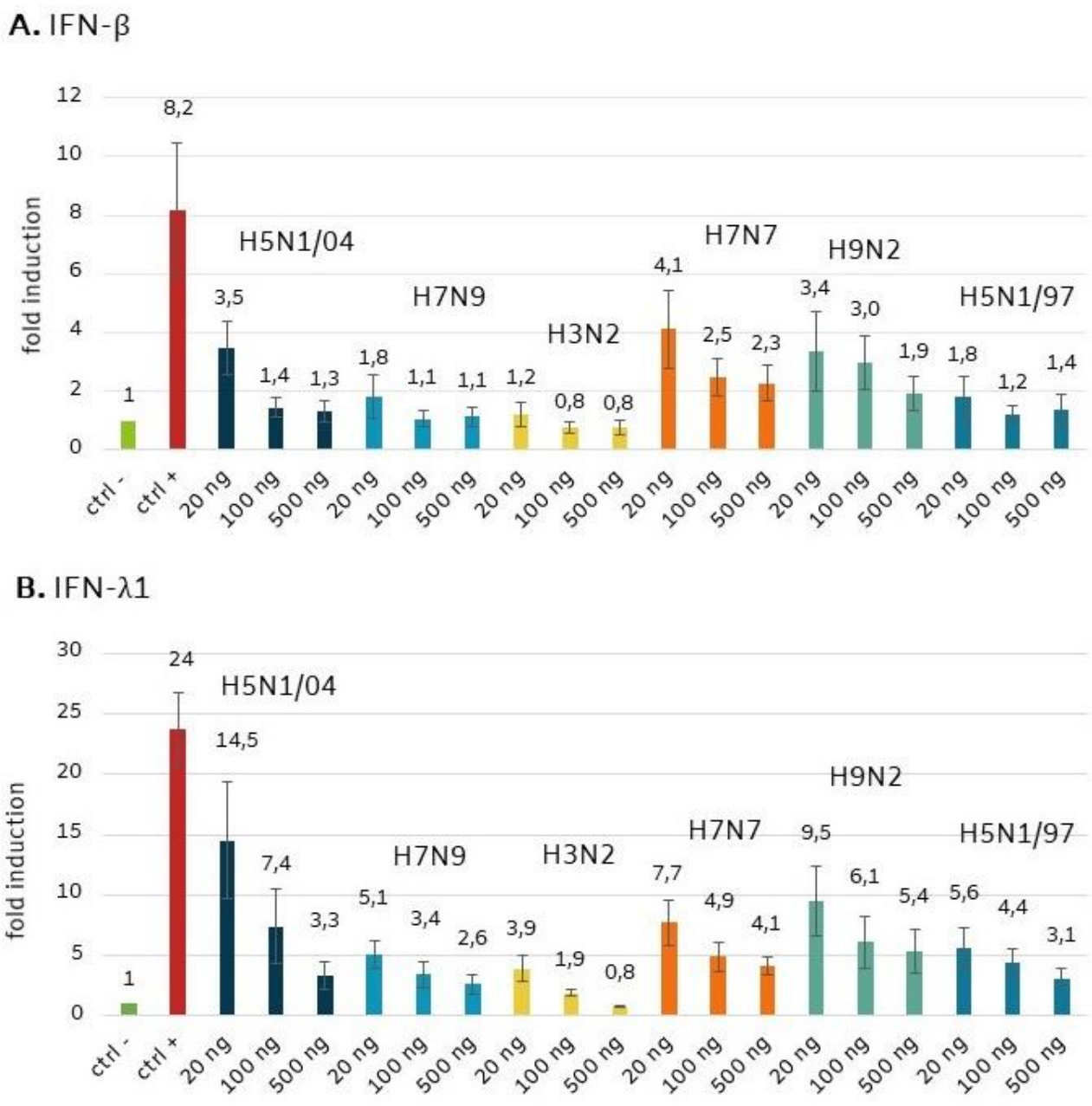


Figure 11. Activation of IFN-β (A) and IFN-λ1 (B) promoter in SV-infected HEK293 cells. Before infection the cells were transfected with a gradient of NS1 protein expression plasmid (20 ng – 100 ng – 500 ng) from several IAV strains: H5N1/04, H7N9, H3N2, H7N7, H9N2 and H5N1/97. Ctrl - samples were transfected only with Lluc and Rluc plasmids and with an empty plasmid without NS1 gene. Ctrl + samples were additionally infected with SV. Cells were collected after 24 h incubation and lysed, and the luminescence was measured. Fold induction was calculated based on CPS values + standard deviation. The graphs show an average fold induction from 5 experiments.

6. Discussion

NS1 plays an important role, not only in the regulation of type I and III IFNs induction and inhibition of ISGs, but also in various other processes influencing viral replication and pathogenicity. It has been shown that changes in NS1 sequence that affect IFN gene expression during the infection have an effect on virulence, host range and tissue tropism of the strain (Ma et al., 2010; Kanrai et al., 2016). For example, D92E substitution in NS1 of H5N1 viruses resulted in more efficient replication in chicken and mice (Seo et al., 2002).

H3N2 suppressed the promoter activation the most for both IFN-β and IFN-λ1 and usually the infection manifests itself rather mildly. Similar results were obtained by Hsu (2011), where H3N2 has suppressed immune response with greater efficiency than H5N1 subtype in human lung cells. The reason for the efficiency of its NS1 in IFN promoter suppression may be the fact that the virus is already adapted to infecting humans and evading the immune response. There are studies which imply that as H1N1 circulates in humans, it slowly regains its ability to supress IFN expression, also suggests that adaptation may be the reason for the high IFN suppression (Clark et al., 2017). Even though the role of NS1 in adaptation to other species has not been established yet, Basler and co-workers has shown that a mouse strain of IAV transformed to express human NS1 of H1N1 Spanish flu virus was not able to replicate in mice (Basler et al., 2001). On the other hand, mouse IAV strain expressing human NS1 was able to replicate more efficiently in human lung cells then the wild type (Geiss et al., 2002).

Second highest IFN-λ1 and IFN-β promoter suppression was observed for NS1 from H7N9. This aligns with the results reported by Arilahti (2014), where H7N9 infection was characterized with lower IFN induction in monocyte-derived dendritic cells than seasonal H3N2 virus, but replicated as well as H5N1 strain. Similar observations were made by Josset et al., where H7N9 shown reduced proinflammatory cytokine production when compared to H3N2 (Josset et al.,2014). This may suggest H7N9 has a higher potential for adaptation to human host.

Even though in this study NS1 of H7N9 shown very high IFN promoter suppression, it was not higher than in presence of NS1 of H3N2 strain. This may suggest that NS1 is not alone responsible for the difference in IFN induction, other factors are involved in the regulation instead. An example may be PA-X protein, which, like NS1, can also influence IFNs production, but uses a different mechanism of action. There are also multiple other

mechanisms in addition to IFN promoter suppression. One of them is inhibition of pre-mRNA processing, including IFN mRNAs. (Nogales et al., 2018).

Out of the studied strains, H5N1/04 and H5N1/97, which cause the most severe symptoms and high death rates in humans, seemed to be efficient at suppressing IFN-β promoter activation. These results did not exactly reflect what was shown by Arilahti (2014), where H5N1/04 infection generated a cytokine storm. H5N1 was previously shown to induce stronger IFN-β and IFN-λ1 response through higher activation of IRF3 compared to seasonal H1N1 virus (Hui, 2009). However, it should be noted that during a viral infection, initially, the NS1 is not present in marked amounts in the cell, it is produced when viral genes are expressed. In this experiment the IFN promoter suppression may have taken place earlier than it would occur during viral infection. In addition to that, only IFN promoter activation, not IFN expression, was measured.

When it comes to IFN-λ1 promoter, H5N1/04 was the least sufficient in suppression of IFN promoter activation. Previous H5N1 virus outbreaks has demonstrated that excessive cytokine production may lead to lung damage, hence severity of the infection and high mortality rates (Kuiken et al., 2012; Zeng et al., 2012). Surprisingly, efficiency in IFN-λ1 promoter suppression of H5N1/97 resembled the results obtained for NS1 of H9N2. Studies conducted by Seo and colleagues has suggested that H5N1/97 are resistant to the effects of IFNs: transformation of human IAV strain with NS1 of H5N1/97 was able to replicate in presence of IFNs, unlike the human strain, which replication was inhibited. (Seo et al., 2002). Other research has also shown that H5N1/97 infection heavily induces proinflammatory cytokines and reduces production of anti-inflammatory cytokines. The imbalance between those two was proposed to be responsible for the severity of symptoms during the infection (Lipatov et al., 2005).

H7N7 and H9N2 are associated with mild or lack of symptoms and their NS1 shown quite low efficiency at suppressing both IFN-β and IFN-λ1. In Westenius (2014) study, H9N2 virus infection was also shown to induce strong IFN genes expression. However, the virus was very sensitive to the effects of type I IFNs, hence explaining the relatively mild course of infection. H7N7 viruses lacking NS1 are attenuated in chickens. However, the main role of NS1 seems to be inhibition of antiviral state and not necessarily supressing IFN genes expression, since the level of IFNs without functioning NS1 was not increased compared than in wild type (Penski et al., 2011).

This study has given some insight into how efficiently potentially pandemic AIV strains can inhibit IFN response, and thus prolong the infection. Even though a definitive correlation between NS1 of various strains and the symptoms caused by them cannot be drawn, these results may be a base for further studies at a deeper mechanistical level, for example which steps of RIG-I pathway are key for suppression of IFN promoter activation by chosen AIVs. The results of the thesis may also be further validated by assessing the expression level of IFN mRNA with qPCR or produced IFN proteins with ELISA test.

Currently, more research is needed to fully determine the relevance of NS1-dependent suppression of IFN promoter activation in pathogenesis of the virus. A better understanding of factors influencing host immune response could improve clinician's ability to control it and increase the survival of the patient.

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